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ON-LINE CAPILLARY ZONE ELECTROPHORESIS–ION SPRAY TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF DYNORPHINS

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SUMMARY

Capillary zone electrophoresis–mass spectrometry and capillary zone electrophoresis–tandem mass spectrometry with ionization at atmospheric pressure are demonstrated as being feasible for the separation and determination of small peptides such as dynorphins (1–6, 1–7, 1–8, 1–9) and leucine enkephalin at low picomole levels by full-scan mass spectrometry and tandem mass spectrometry and at the low femtomole range under selected ion monitoring conditions. Ion evaporation resulting from the ion spray liquid chromatography–mass spectrometry interface exhibits primarily molecular weight information as singly and multiply charged ions and is shown to be a sensitive and mild ionization method for peptides. The full-scan daughter ion mass spectrum of leucine enkephalin is shown to contain fragment ions consistent with the sequence of the peptide. Parent ion scanning in the tandem mass spectrometry mode is a promising technique for the screening of related peptides.

INTRODUCTION

About 10 years ago, one of the classical papers introducing high-performance electrophoresis as the electrophoretic counterpart of high-performance liquid chromatography (HPLC)[1] confronted its readers with the following statement: "Many of the problems in the development of electrophoresis can be reduced to convection and detection". The concept of high-performance capillary electrophoresis[2], for which the most commonly known mode of operation is capillary zone electrophoresis (CZE)[3], includes the alleviation of the convection problem by using narrow-bore open tubes as the separation chamber, thereby allowing rapid dissipation of the joule heat generated by the applied electric field. This approach guarantees stable electrophoresis performance owing to the "anti-convective wall-effect"[1], in contrast to the more

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common solution of using such anti-convective stabilizers as paper, cellulose acetate and gels to suppress convective zone broadening in electrophoresis techniques. This approach also brought about the realization of a powerful instrumental version of electrophoresis[4], analogous to modern column chromatography, with its appreciated high standard of automation. Recently published studies focusing on different aspects of CZE instrumentation still emphasize this key concern for present and future developments[5–8].

With regard to the above-mentioned detection problem, the principle of on-column detection feasibility in CZE again opened new possibilities for analyte characterization. The readily installed optical detectors based on UV absorption and fluorescence emission[9,10] were soon followed by other techniques promising to fulfil the requirements of universal detection on the one hand and high sensitivity on the other. This capability is especially challenging owing to detection volumes in the low nanoliter to picoliter range[11,12].

Smith and co-workers[13,14] were the first to report the successful interfacing of CZE with mass spectrometry (CZE–MS). Their interface was based on the electrospray ionization liquid interface for mass spectrometry developed by Whitehouse *et al.*[15]. There should be little need here to review the merits of mass spectrometric detection with regard to universality, high sensitivity and outstanding selectivity. The success of gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography–mass spectrometry (HPLC–MS) easily attests to the importance of the mass spectrometer as the preferred detector for modern on-line separation techniques.

Although HPLC, another powerful separation technique, has become more widely used in the biochemical field in the last decade owing to advances in instrumentation and automation, there is no doubt that electrophoresis in its various forms is still the premier method for the separation and analysis of peptides, proteins and polynucleotides. The impact of mass spectrometry on the analytical demands in the life sciences has become increasingly important[16–19]. Research in the area of endogenous peptides, for example, has frequently profited from progress in mass spectrometry[20] since the successful identification of enkephalins in brain tissues by Hughes *et al.*[21]. An excellent overview of state-of-the-art mass spectrometric determination of peptides and proteins was recently published by Biemann and Martin[22].

The potential of CZE to separate peptides, proteins and polynucleotides has already been demonstrated[3,23,24]. The new technique of CZE–MS, which combines the separation efficiency of electrophoresis for charged species with the well known capability of mass spectrometry for structure elucidation, should be well suited for the analysis of complex mixtures of charged biochemical compounds.

Here we describe in brief the instrumental and experimental aspects of our approach to on-line CZE–MS coupling, which will be presented in more detail elsewhere[25]. We also present results demonstrating the feasibility of this technique for the determination of small peptides.

EXPERIMENTAL

Electrophoresis

The capillary electrophoresis system consisted of a 0–60 kV voltage regulated

power supply (Spellman, Model RHR60P30/EI, Plainview, NY, U.S.A.). The high-voltage lead was connected directly to a 2-ml carbon vessel which served a dual purpose as buffer reservoir and electrode. The separation column, a 100 cm × 100 μm I.D. length of uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, U.S.A.), was suction-filled with buffer and the anode end of the column was suspended in the carbon vessel containing the buffer medium. The cathode end of the column was connected to the ion spray LC–MS interface[26] via a liquid junction coupling described in more detail elsewhere[25]. The buffer used was acetonitrile–30 m*M* acetate buffer (pH 4.8) (1:1). Sample volumes of 2 to 30 nl were introduced into the column at the anode end by hydrostatic injection (siphoning)[27]. The injection volume was determined by injecting a narrow band of a dark dye and measuring the time required for it to pass through a given length of the column while in the inject mode. A potential of 30 kV was applied to the anode end of the separation column through the carbon buffer reservoir and 3 kV was applied to the cathode end through the ion spray interface, which produced a net CZE voltage of 27 kV. A CZE voltage of 27 kV was used for all separations reported and the current (20–40 μA) passing through the CZE column was measured with an in-line analog ammeter (Model 260; Simpson, Chicago, IL, U.S.A.).

Mass spectrometry

A SCIEX TAGA 6000E triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization (API) source was used to sample ions produced from the ion spray LC–MS interface. In this system ions in the gas phase are drawn into the vacuum of the mass spectrometer through a 100 μm I.D. orifice. The atmospheric side of the orifice is bathed with a curtain of high-purity, dry nitrogen gas. The nitrogen curtain acts as a barrier that restricts contaminants and solvent vapor from entering the mass spectrometer vacuum. High vacuum in the analyzer region of the mass spectrometer is achieved by cryogenically cooled surfaces, maintained at 19–20 K, surrounding the quadrupoles. During routine operation the indicated vacuum was $7 \cdot 10^{-6}$ Torr and during collision-induced dissociation (CID) the vacuum was $1.5 \cdot 10^{-5}$ Torr with a target gas thickness of $230 \cdot 10^{12}$ atoms/cm^2 of argon in the collision cell. A collision energy of 50 V was used for all CID experiments.

Chemicals

The peptides dynorphin 1–6, 1–7, 1–8 and 1–9 and leucine enkephalin (dynorphin 1–5) were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification. HPLC-grade ammonium acetate, glacial acetic acid, water and acetonitrile were obtained from Fisher (Rochester, NY, U.S.A.).

RESULTS AND DISCUSSION

The injection reproducibility for the system described above was determined by triplicate 10-s hydrostatic injections at a height of 5 cm for three different concentrations of leucine enkephalin (Fig. 1). As the flow of the CZE system can be stopped during operation by simply turning off the high voltage, serial injections can be made at close intervals. Amounts of 24, 120 and 600 fmole of leucine enkephalin were

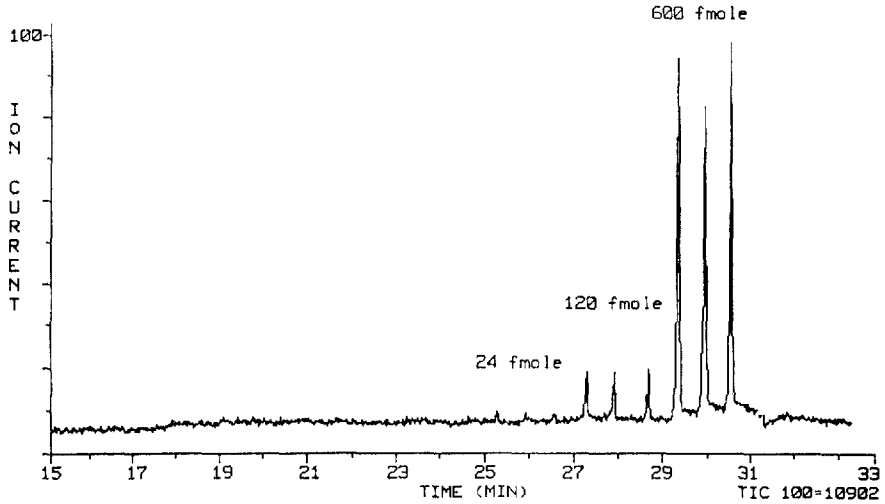

Fig. 1. SIM CZE–MS electropherogram for repetitive triplicate injections of 24, 120 and 600 fmole levels of leucine enkephalin. The $(M+H)^+$ ion at m/z 556 was monitored.

injected with mass spectral acquisition of its protonated molecular ion at m/z 556. The peak widths at half-height for all peaks in the serial injection electropherogram (Fig. 1) were measured and found to be the same (1.2 mm). This demonstrates that band broadening from each injection was not observed. The peak heights and areas

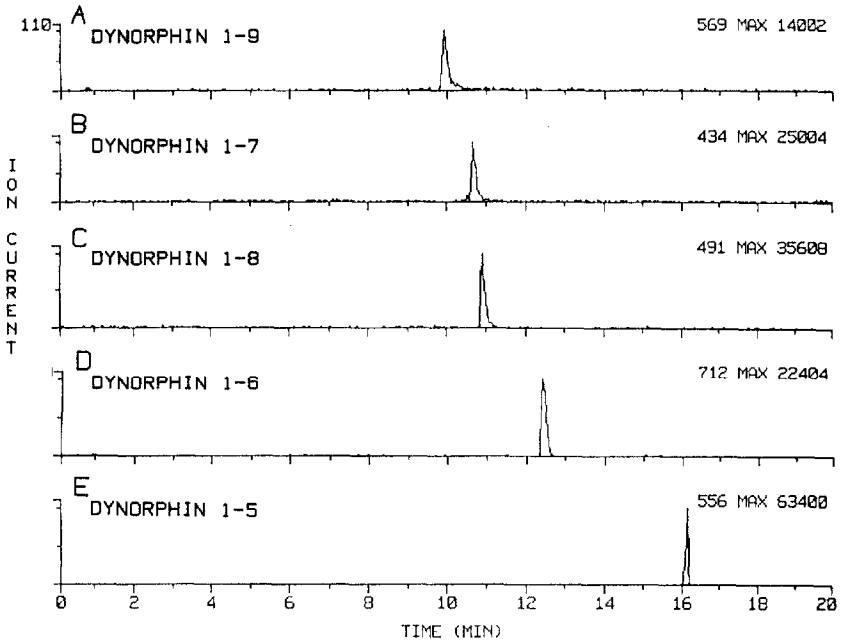

Fig. 2. Full-scan CZE–MS extracted ion electropherograms for 5 pmole per component of (A) dynorphin 1–9, (B) dynorphin 1–7, (C) dynorphin 1–8 (D) dynorphin 1–6 and (E) leucine enkephalin (dynorphin 1–5).

within each triplicate (Fig. 1) are within 10% of the other two peaks, demonstrating satisfactory reproducibility from the system. All injections were made by manual operation. If this system were automated it should follow that reproducibility would be within 1–2%.

A CZE separation was obtained for 5 pmole each of dynorphin 1–9, 1–7, 1–8 and 1–6 and leucine enkephalin under full-scan CZE-MS conditions (Fig. 2). The mass spectrometer was scanned from m/z 300 to 750 at a rate of 2 s per scan. The

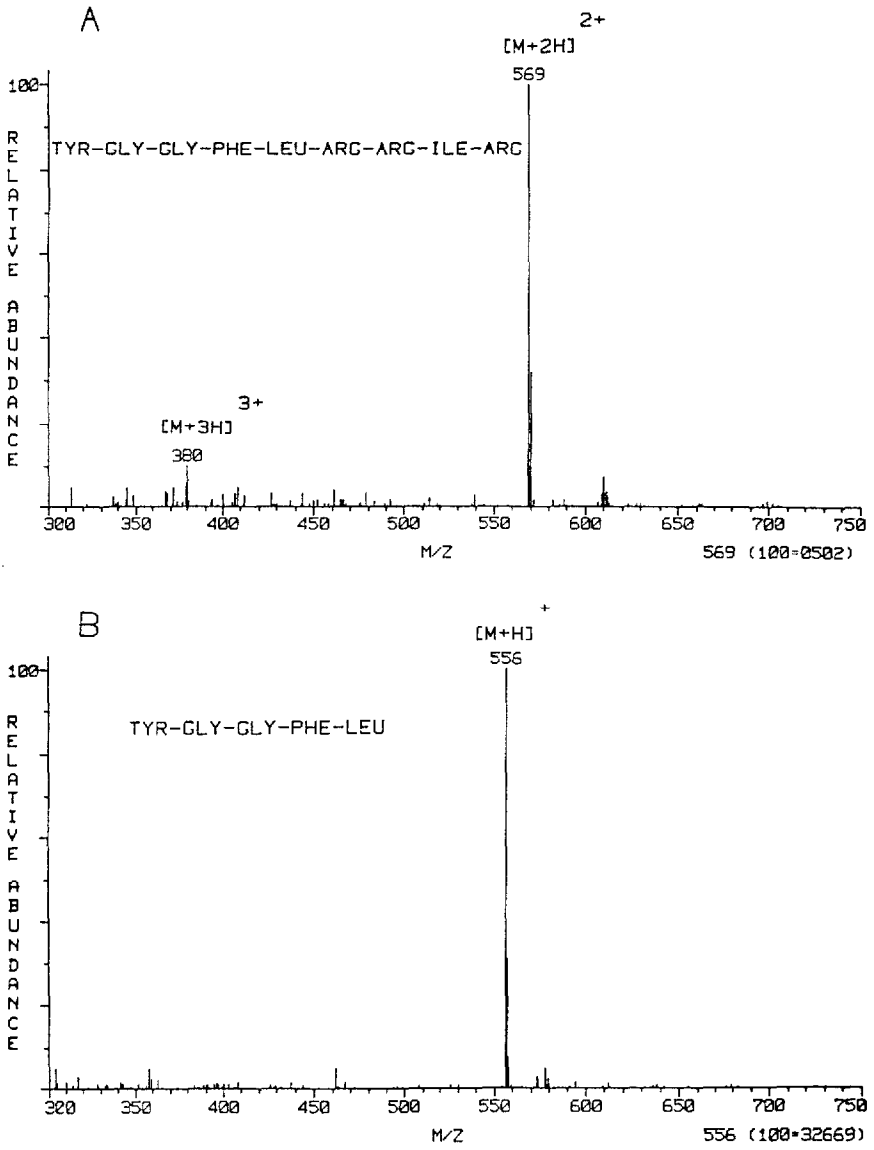

Fig. 3. CZE–MS mass spectra for (A) dynorphin 1–9 and (B) leucine enkephalin from Fig. 2. Ions are formed by ion evaporation at atmospheric pressure via the ion spray LC–MS interface.

individual extracted ion current electropherograms from this CZE–MS experiment show good separation for all five components. Because the capillary electrophoresis separation was developed with the anode end at high positive voltage (30 kV) and the cathode at low voltage (3 kV), the net electroosmotic flow of the system was from the anode to the cathode and species with more positive electrophoretic mobilities were eluted first. Electrophoretic mobility is a function of charge and size, and the more positively charged species should be eluted first[3]. As shown in the corresponding CZE–MS mass spectra (Fig. 3), obtained from the electropherogram shown in Fig. 2, the mass spectrum of the first-eluted peak, dynorphin 1–9 (Fig. 3A), contains both triply protonated, triply charged (*m/z* 380) and doubly protonated, doubly charged (*m/z* 569) molecular ions and the last-eluted peak, leucine enkephalin (Fig. 3B) contains only the singly charged, protonated molecular ion (*m/z* 556). Under the acidic CZE conditions used the charge on each peptide in solution is a function of the number of terminal amino functionalities. This is evidenced by the fact that dynorphin 1–7 (Fig. 2B) is eluted before dynorphin 1–8 (Fig. 2C). Both dynorphin 1–7 and dynorphin 1–8 contain three terminal amino groups; however, the mass of dynorphin 1–8 is greater. Therefore, dynorphin 1–8 has a lower mobility than dynorphin 1–7, so it is eluted later. As can be seen, the elution order is a function of the charge and size of the species with the most positively charged and small size species being eluted first.

The ion spray LC–MS interface utilizes a form of electrospray ionization where gaseous ions are produced by the mechanism of ion evaporation[28]. This is a mild form of ionization where predominantly singly protonated, singly charged and/or multiply protonated, multiply charged molecular ions are formed. This provides an opportunity for operating the mass spectrometer in the selected ion monitoring (SIM) mode with high sensitivity, as the ion current is concentrated into only a few species which are characteristic of the analyte. By operating in the SIM mode lower CZE–MS detection limits can be achieved with higher separation efficiencies. This is evidenced in the SIM electropherogram (Fig. 4) for 2 pmole per component of the mixture

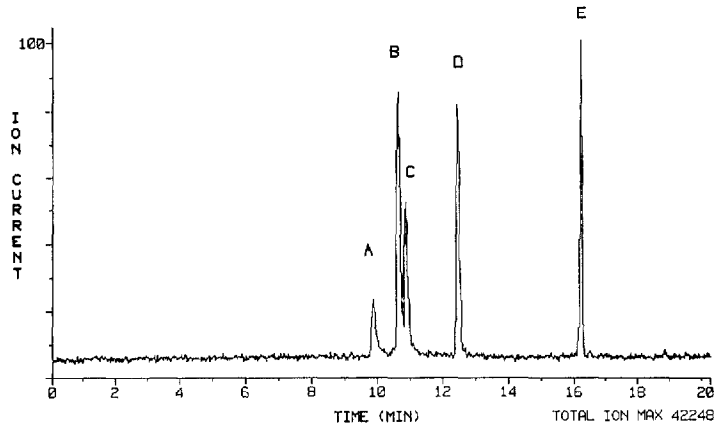

Fig. 4. Total SIM CZE–MS ion electropherogram for 2 pmole per component of (A) dynorphin 1–9 (*m/z* 380 and 569), (B) dynorphin 1–7 (*m/z* 434), (C) dynorphin 1–8 (*m/z* 491), (D) dynorphin 1–6, (*m/z* 356 and 712) and (E) leucine enkephalin (*m/z* 556).

described in Fig. 2, where the last-eluted peak (E) exhibits efficiencies in excess of 250 000 theoretical plates.

Another advantage of having the molecular ion carry most of the ion current is that sensitivity is increased for tandem mass spectrometry (MS–MS) experiments. By monitoring the protonated molecular ion of leucine enkephalin at *m/z* 556 with the first quadrupole, performing collision-induced dissociation (CID) in the second qua-

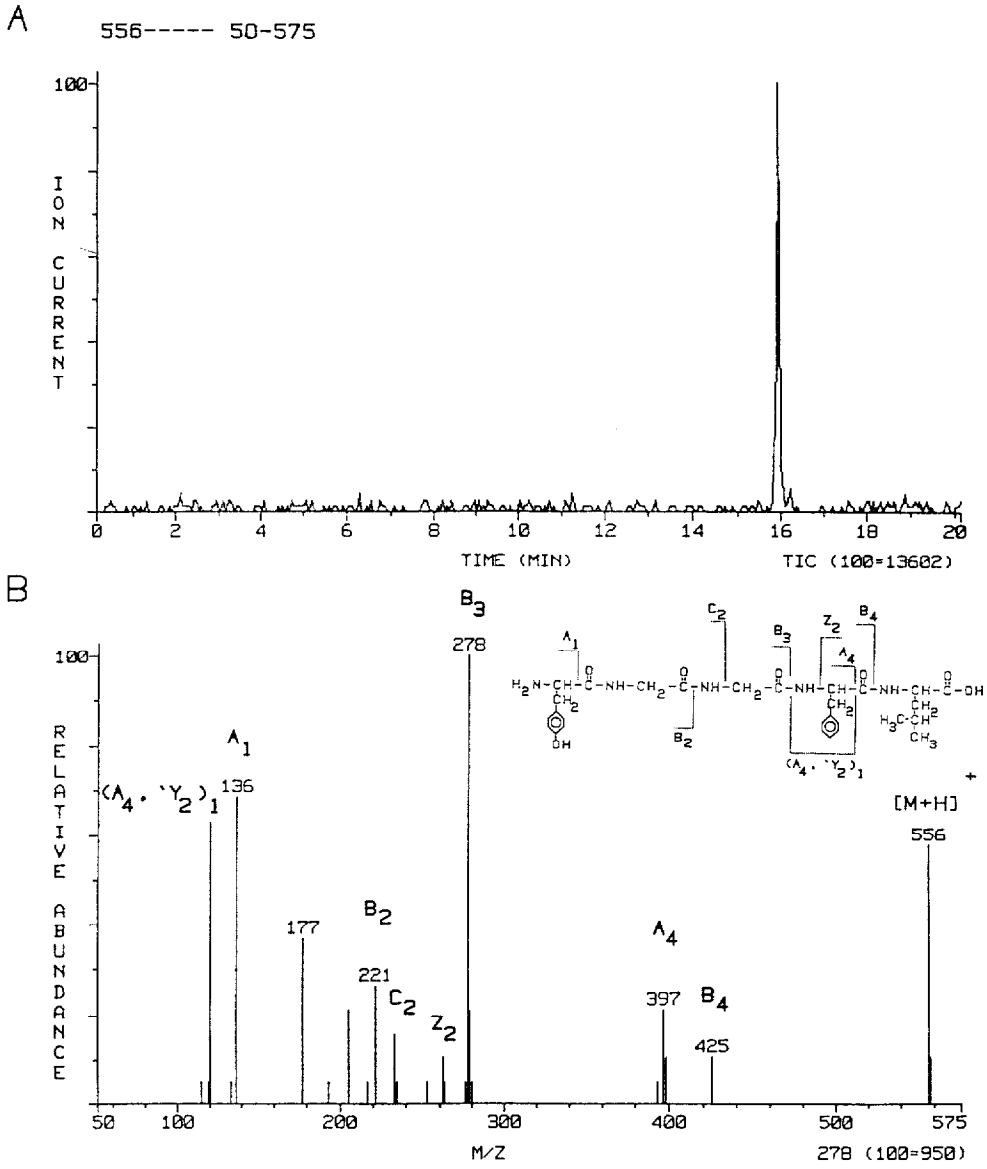

Fig. 5. (A) Full-scan CZE–MS–MS (from *m/z* 50 to 556) total daughter ion current electropherogram and (B) collision-induced-dissociation mass spectrum for 5 pmole of leucine enkephalin. The parent ion for CID was $(M+H)^+$ ion at *m/z* 556.

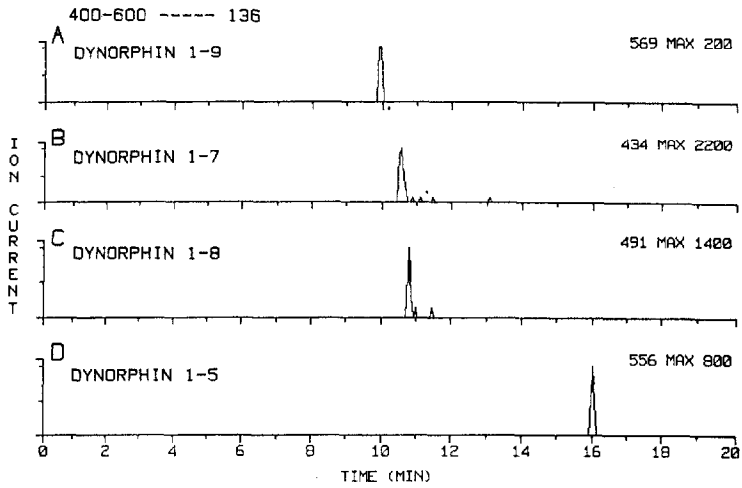

Fig. 6. CZE–MS–MS extracted parent ion electropherograms for the common daughter ion at *m/z* 136 for (A) dynorphin 1–9, (B) dynorphin 1–7, (C) dynorphin 1–8 and (D) leucine enkephalin. Quadrupole 1 was scanned from *m/z* 400 to 600 and Quadrupole 3 monitored *m/z* 136.

drupole, and scanning the third quadrupole from *m/z* 50 to 556 the total ion electropherogram (Fig. 5A) and the daughter ion mass spectrum (Fig. 5B) for 5 pmole of leucine enkephalin were acquired[29]. The daughter ion mass spectrum (Fig. 5B) is consistent with the sequence of the pentapeptide, leucine enkephalin, and provides the necessary information required to identify its structure. The nomenclature scheme proposed by Roepstorff and Fohlman[30] was used to label the sequence ions of the CID daughter ion mass spectrum of leucine enkephalin (Fig. 5B). The daughter ions B_2, Z_2, B_3, A_4 and B_4 are consistent with fragment ions from fast atom bombardment linked-field scanning of leucine enkephalin reported in the literature[31].

Because $(A_4, {}^{\prime}Y_2)_1$ at *m/z* 120 and A_1 at *m/z* 136 are common CID daughter ions of all dynorphins, parent ion scans can be performed as a screen for possible components in a mixture[29]. The parent ion experiment (Fig. 6) for 5 pmole per component of dynorphin 1–9, 1–7 and 1–8 and leucine enkephalin was performed by scanning the first quadrupole from *m/z* 400 to 600 and monitoring *m/z* 136 in the third quadrupole. Dynorphin 1–9, 1–7 and 1–8 were identified by their doubly protonated, doubly charged molecular ions at *m/z* 569, 434 and 491, respectively, and leucine enkephalin was identified by its singly protonated, singly charged molecular ion at *m/z* 556.

CONCLUSIONS

These results demonstrate the feasibility of using CZE–MS and CZE–MS–MS with an atmospheric pressure ionization (API) triple quadrupole mass spectrometer for the determination of small peptides. CZE is capable of separating small peptides with high efficiencies, from 50 000 to 250 000 theorectical plates, relatively rapidly. The ion spray LC–MS interface is well suited for CZE and API mass spectrometry owing to its high sensitivity for species which exist charged in solution. Quantitation

with this system should be possible owing to the satisfactory reproducibility of injection. CZE–MS–MS complements CZE–MS by producing additional structural and sequence information. At present, concentrations of the analytes of interest in the sample solution are still in the high parts per billion to low parts per million range. Improved sensitivity of the mass spectrometer and higher sample capacity of the CZE column would improve the utility of the combined CZE–MS system. Future efforts should be directed towards these goals.

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